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13. ABSTRACT *(Maximum 200)*

We studied developmental changes in glucose transporter (GT) targeting in mammary gland, a prerequisite for the understanding GT targeting in breast cancer. Previously, we established that GLUT1 is targeted to Golgi during lactation. To understand the regulation of this process, we carried out subcellular fractionation and density gradient centrifugation, Western blotting, and immunofluorescence in mammary glands of mothers whose pups were prematurely weaned. We conclude: 1) There is enrichment of Golgi by GLUT1 during lactation. This is lost by 5h of weaning. 2) Enrichment can be restored by returning the pups to the mother for 5h. 3) At 10h, total cellular content of GLUT1 begins to decrease. 4) A 72 kD protein recognized by the GLUT1 antibody showed even more striking Golgi enrichment than GLUT1. 5) Intermediate MW forms at 50 and 65 kD were also observed. These each demonstrate specific patterns of appearance, disappearance, and subcellular localization, and can be deglycosylated to give aglycoGLUT1. 6) The 72 kD protein was resistant to deglycosylation. Based on the kinetics of its appearance and disappearance, its physicochemical properties which suggest it is not a GT, and based on its subcellular localization in the Golgi, p72 is an excellent candidate for a protein involved in sequestering GTs within the Golgi. 7) Ubiquitin appears to play an important role in the rapid degradation of GLUT1 and p72 during premature weaning. Differential display analysis of gene expression regulated by prolactin and dexamethasone has identified five novel genes, and full length clones are being isolated. One of these may encode a protein with a motif similar to the trans-Golgi targeting motif of the GLUT4 GT.

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FOREWORD

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Peter M Hawley MD PhD 10/17/97

PI - Signature Date

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Introduction-

Prologue-

As of July 1, 1997, I became an Assistant Professor of Pediatrics at the Baylor College of Medicine in Houston Texas. I am affiliated with the Section of Neonatology and with Texas Children's Hospital and my lab is in the U.S.D.A./A.R.S. Children's Nutrition Research Center.

This move reflects my commitment to studying mammary gland biology and breast cancer. It has resulted in a major increase in the resources, personnel, and collaborators and consultants available to me. The CNRC offers excellent facilities and 35 other investigators pursuing aspects of maternal and child nutrition, including lactation and mammary gland biology, as well as epithelial cell biology. The Department of Cell Biology at the Baylor College of Medicine is open and welcoming, has a major research interest in the mammary gland, and is a major site of DOD-sponsored breast cancer research. The M.D. Anderson Cancer Center, another major site of breast cancer research, is also nearby in the Texas Medical Center. The clinical facilities of the Baylor College of Medicine offer large volumes of clinical material. The core facilities at Baylor are outstanding and are facilitating several aspects of the project. In addition to the technician which this grant funds, I now have a second technician as well. One of the first year neonatology fellows has chosen to spend two years doing research in my lab. The institutional support at Baylor is superior to what I enjoyed at Washington University. The net effect of this move on my productivity should be extremely positive.

Of course, the move from St. Louis to Houston resulted in discontinuity in the research effort, as work needed to wind down in St. Louis, and new technicians needed training in Houston. Nevertheless, I am reporting progress on studies of the weaning period. These studies show remarkably rapid and reversible changes in glucose transporter targeting. Taken together with work that I reported last year, we are well along with the first task of the Statement of Work, describing developmental changes in glucose transport in mammary gland. I anticipate no difficulty in concluding this within five more months, within the parameters of the S.O.W. The second task of my S.O.W., the identification of novel proteins involved in glucose transport, has required much effort during the past year in pursuing several apparently novel proteins. This difficult work and the progress we have made is reported. Preliminary work has begun on the third task of the S.O.W., the study of glucose transporters in cancer cells, but I am not presenting any data regarding that task in this report. Although the S.O.W. called for that work to begin six months ago, extra efforts pursuing tasks 1 and 2 and moving from St. Louis to Houston required me to defer this by five months. Nevertheless, for the reasons outlined in the second paragraph, I anticipate significant progress during the next year in achieving this task.

Note that, since DOD policy did not allow the grant to be transferred to Baylor College of Medicine, F. Sessions Cole, M.D. graciously agreed to serve as Principal

Investigator and to subcontract the work to me at Baylor College of Medicine. Therefore, I am technically submitting this report on his behalf.

a. the subject of the research

Glucose is critical to mammary epithelial cells not only because it serves as a fuel and as a building block for glycoproteins and glycolipids, but also because of its role as the major substrate for the synthesis of lactose and lipid, which together contribute 80-90% of the calories in human milk. Lactose is the major carbohydrate constituent of human milk and the major determinant of its osmolarity, and therefore, of milk production. Synthesis of lactose is carried out exclusively within the Golgi apparatus of mammary epithelial cells, in a reaction catalyzed by galactosyltransferase complexed to the tissue-specific protein alpha-lactalbumin(Strous, 1986). Lipid components of human milk, which are also primarily derived from glucose, provide about one-half of the caloric content of milk. Thus, regulation of glucose uptake in mammary epithelia must account for two very different states, the quiescient state, with a relatively small demand for glucose, and the lactating state, with an extraordinary demand for glucose to fuel the high metabolic rate of the epithelial cells themselves as well as to provide substrate for the synthesis of milk to sustain the young.

Breast cancer cells also exhibit an increased demand for glucose, as reviewed below. The molecular mechanisms by which the enhanced transport of this vital nutrient into tumor cells is accomplished require further investigation. Elucidation of the molecular mechanisms by which the mammary epithelia achieves the adaptations in glucose transport needed for lactation, and the examination of their possible dysregulation in neoplastic mammary epithelium, form the central goals of this proposal.

b. the background of previous work

There are two mechanisms for glucose transport into cells. For most cells, the sole mechanism is the passive diffusion of glucose into cells, facilitated by the five isoforms of the glucose transporter family(Burant, et al., 1992, Mueckler, 1994). These are designated GLUT1-GLUT4, and GLUT7, in the order in which they were cloned. (GLUT5 is actually a fructose transporter(Burant, et al., 1992), and GLUT6 is a pseudogene related to GLUT5(Kayano, et al., 1990)). These isoforms exhibit distinct regulatory properties, tissue distributions, and kinetics. However, they are all integral membrane proteins containing approximately 500 amino acids. Hydropathy plots based on amino acid sequences predicted from cDNA sequence suggest that all share a common topology, which includes cytoplasmic N- and C-termini, twelve membrane spanning domains, one exofacial loop which contains an N-linked glycosylation site, and one approximately 65 amino acid intracellular loop midway through the transporter. There is a striking degree of homology among these isoforms, which are 50-65% identical in their amino acid sequence. GLUT1 is also known as the endogenous glucose transporter because of its nearly ubiquitous tissue distribution. It is important in basal glucose uptake and is usually found primarily in the plasma membrane. It is

the only glucose transporter isoform convincingly shown to be expressed in mammary epithelia. In the specialized setting of intestinal(Hwang, et al., 1991) and renal(Miller, et al., 1992, Pajor, 1994) epithelia, and possibly in pulmonary epithelia(Kemp and Boyd, 1992), glucose is also taken up by active transport across specialized membrane domains by tissue-specific isoforms of the sodium, glucose co-transporter(Hediger, et al., 1987). A possible role of this protein in mammary gland glucose transport during lactation has recently been suggested(Shennan and Beechey, 1995).

Mammary gland is unique in its requirement for free glucose within the Golgi, the site of lactose synthesis from glucose and UDP-galactose. The substrates for glycosylation of proteins within the Golgi, which occurs in many cell types, are nucleotide sugars, not free sugars. Wilde and Kuhn measured glucose uptake into rat mammary acini at different glucose concentrations, and directly measured intracellular glucose concentration, to conclude that glucose transport is rate-limiting for lactose synthesis(Wilde and Kuhn, 1981). Madon et al. measured cytochalsin B binding of fractionated rat mammary gland and found that the GLUT1 glucose transporter accounted for only about one-half of the cytochalasin B binding sites of Golgi, strongly suggesting that a unique transporter resides in the Golgi of lactating mammary gland(Madon, et al., 1990). This proposal aims to extend our understanding of glucose transport within lactating mammary gland by identifying and characterizing the molecular species responsible for glucose transport within the mammary epithelial cell and by exploring their developmental and hormonal regulation. This forms a prerequisite for understanding glucose transport in breast cancer.

Seven decades ago, Warburg(Warburg, 1923) appreciated that tumor cells show high rates of glucose uptake, glucose metabolism, and respiration(Hatanaka, 1974, Merrall, et al., 1993). Several lines of evidence suggest the value of a comprehensive understanding of glucose transport in mammary gland in the context of breast cancer. Brown et al.(Brown and Wahl, 1993) showed that higher expression of the glucose transporter GLUT1 by breast cancer cells compared with the healthy breast tissue is common. Several groups have recently shown that the glucose analog 18-F-fluoro-2-deoxyglucose can be used to detect and stage breast cancer(Wahl, et al., 1991, Tse, et al., 1992, Nieweg, et al., 1993, Adler, et al., 1993, Crowe, et al., 1994), suggesting that an abnormally high uptake of glucose is a consistent finding in breast cancer. The MCF-7 line of breast cancer cells was established over twenty years ago(Soule, et al., 1973) and has been characterized in literally hundreds of studies since then. Inhibition of glycolysis in MCF-7 breast cancer cells by extracellular AMP markedly inhibited cell proliferation(Hugo, et al., 1992). Elegant NMR studies have recently shown that, in MCF-7 human breast cancer cells, tamoxifen inhibits glucose consumption and lactate production by 50%, compared to estrogen-treated cells, and that estrogen rescue of tamoxifen treated cells was associated with a rapid increase in glucose consumption(Furman, et al., 1992, Neeman and Degani, 1989). Glucose-6-phosphate dehydrogenase, a key enzyme of glucose metabolism, is strikingly elevated in mammary epithelial cells from patients with breast cancer compared to those with

benign breast disease(Barron, et al., 1991). Its activity was also significantly increased in morphologically normal tissue from cancer-containing breasts when compared to breasts with no known cancer, suggesting the possibility that metabolic abnormalities precede morphological changes in breast carcinogenesis(McDermott, et al., 1990). GLUT1 is the major glucose transporter isoform expressed in mammary epithelial cells. Importantly, GLUT1 is also the only known glucose transporter isoform whose gene is activated at the level of transcription in cells transformed by oncogenes such as *fps* , *src* , and *ras* (Birnbaum, et al., 1987, Flier, et al., 1987). Since this response is direct, the GLUT1 gene is an immediate early gene. Of special interest with respect to breast cancer is that the *neu* oncogene induces synthesis of GLUT1 mRNA and increases glucose uptake three-fold in fibroblasts(Sistonen, et al., 1989). Several groups have reported that between 18.8% and 67% of patients with breast cancer have elevated serum *neu* protein levels or amplification of HER-2/*neu*.(Kath, et al., 1993, Charpin, et al., 1993, Descotes, et al., 1993, Bacus, et al., 1992). Induction of GLUT1 mRNA synthesis also occurs in cells after addition of serum, peptide growth factors, and agents which increase intracellular cAMP concentration(Hiraki, et al., 1989). The two enhancer elements responsible for the responsiveness of the GLUT1 gene to growth factors and oncogenes have been characterized(Muramiki, et al., 1992).

What might the biochemistry and molecular cell biology of glucose transport in lactating mammary gland teach us about breast cancer? One fundamental answer is that, to understand the abnormal state of a cell, one must first understand its normal function and development. A more concrete rationale is suggested by the observation that high rates of glucose uptake and high levels of GLUT1 characterize breast tumors, as noted above. Furthermore, certain proteins important in lactation are also expressed in neoplastic breast tissue but not in normal, quiescent, breast tissue. Serum human alpha-lactalbumin, the mammary-specific protein cofactor that combines with galactosyltransferase to form the complex lactose synthetase, has been proposed as a marker for breast cancer(Thean and Toh, 1990). Similarly, a milk fat globule protein is highly expressed in human breast tumors(Hilkens, et al., 1986, Larocca, et al., 1991). There is also a higher molecular weight glycoprotein detectable in milk and breast carcinomas(Sekine, et al., 1985). None of these are expressed in non-lactating, non-neoplastic mammary gland.

c. the purpose and scope of the present work

In contrast to the transfer of lactose into milk across apical membrane, which occurs by vesicle fusion and is not carrier-mediated, glucose must be transported across at least three distinct cellular membranes of lactating mammary epithelial cells. Specifically, glucose must be transported from the blood across the basal plasma membrane to the mammary gland cytoplasm, from the cytoplasm across the Golgi membrane to the Golgi, where lactose synthesis occurs(Kuhn and White, 1975), and from the cytoplasm across the apical membrane to milk. No known isoforms of the glucose transporter family are known to reside primarily in the Golgi. The regulation of glucose transport

must take into account the difference in requirements of the quiescent and the lactating gland. Therefore, the specific hypotheses to be tested are:

1. Glucose transport into mammary epithelial cells is subject to a high degree of regulation.

GLUT1 and any other transporter isoforms or other novel proteins identified by pursuing the first specific aim are likely to exhibit developmental and hormonal regulation. The activities of key enzymes of lactation, such as acetyl-CoA-carboxylase, fatty acid synthetase, galactosyltransferase, phosphofructokinase, and isocitrate dehydrogenase, among others, expressed per mg DNA, rise several-fold as lactation commences(Wilde, et al., 1986). It is reasonable to expect coordinate regulation of glucose transporters. Given the crucial importance of successful lactation to continued survival of the species, and given the limited evidence that already exists, I expected to confirm that glucose transport into lactating mammary gland is, in fact, exquisitely regulated, and proposed to establish the molecular mechanisms of that regulation.

2. Glucose transport into mammary epithelial cells involves novel proteins.

Novel glucose transporters with Golgi retention signals may be responsible for glucose transport into Golgi during lactation. GLUT1 is the only known glucose transporter known to be expressed in mammary gland. In all other cell types tested, GLUT1 is targeted to plasma membrane. If GLUT1 is responsible for Golgi glucose transport, a novel tissue- and differentiation-stage specific mechanism must exist to account for this. Therefore, it is in any case likely that novel proteins will be identified.

3. Abnormal glucose transport in mammary epithelial cells (i) is associated with abnormal cell growth, and (ii) may facilitate abnormal cell growth.

Only by comprehensively characterizing glucose transport in normal quiescent and lactating mammary gland will we obtain the data needed to understand the significance of a possible role for altered glucose transport in breast cancer. As noted above, there is substantial evidence linking glucose uptake and metabolism with cancer. I anticipate that the association between glucose metabolism and breast cancer will be confirmed. Using techniques of molecular cell biology, I will be able to test whether specific perturbations of glucose transport, including those which might be found to be associated with cancer, can of themselves confer an abnormal phenotype. I also intend to alter glucose transporter targeting in tumor cells to reduce the amount at the cell surface available for glucose uptake; this will test the hypothesis that abnormally high glucose transport is important to support tumor cell growth. There are currently no data upon which to base a prediction of the results of this experiment, although there is the precedent that the *v-sis* oncoprotein loses transforming activity when targeted to the early Golgi complex(Hart, et al., 1994). Successful reduction of glucose transport into tumor cells might offer new therapeutic possibilities.

In order to test these hypotheses, the following specific aims were chosen:

1. Description of the developmental and hormonal regulation of glucose transport in mammary gland.

In work funded by this grant and reported in previous annual reports, double-label immunofluorescence and subcellular fractionation by density gradient centrifugation were used to demonstrate that GLUT1 is localized, both in vitro and in vivo, in the Golgi in response to the hormonal milieu of lactation. Northern and Western blots for GLUT1 and GLUT5 indicated that the developmental regulation of glucose transporters was isoform-specific, and a rapid decline in GLUT1 levels at weaning was linked to changes in the translational efficiency or increased GLUT1 degradation. GLUT1 has been identified in total plasma membrane and Golgi fractions of lactating rat mammary gland.

Note that I am presenting data from mouse rather than rat. I have chosen to focus on mouse for several reasons. First, the established cell lines are murine. Secondly, the mammary gland transplantation technique I will soon be applying has been used in mice but not rats. Thirdly, normal mouse data would be needed to interpret data from any transgenic or knockout mice I might generate in work beyond the scope of this porposal.

Having established the above in previous years, we focused during this reporting period on precocious weaning, reasoning that information about the rates of GLUT1 retargeting during this period would be useful and would help define the best system for mechanistic studies.

2. Identification of novel proteins involved in glucose transport in lactating mammary epithelia .

Preliminary data show that GLUT1 localizes to Golgi as well as to plasma membrane in lactating mammary gland. The mechanism of this cell-type specific targeting to Golgi is unknown. I hypothesize that a tissue-specific sorter must interact with GLUT1 to accomplish this. This interaction must depend on a unique structural determinant of GLUT1, which would constitute a hormonally regulated Golgi targeting motif. Identification of the unique GLUT1 structural determinant will provide a tool for the identification of the sorter.

Progress during the last year, reported here, identifies the protein denoted p72 as an initial candidate for a GLUT1 sorter. This protein is localized to Golgi and degrades rapidly upon premature weaning. Its rapid degradation is due to its highly specific conjugation with ubiquitin. This 72kD protein contains the C-terminal epitope of GLUT1. p72 is not a glycoprotein, and is presumably about 200 amino acids larger

than the glucose transporter isoforms. It is therefore quite unlikely that p72 is itself a glucose transporter. There is evidence that GLUT1 forms oligomers (Pessino, et al., 1991), although the structural determinants of association have not yet been worked out. I speculate that p72 associates with GLUT1 to alter its targeting, causing an increase in the steady-state distribution of GLUT1 in Golgi.

As discussed in last year's report, we are focusing on differential display of genes expressed under regulation of prolactin and dexamethasone as a major approach to this task, and progress is reported below. I discuss in the proposal and list on the Statement of Work several other strategies available to us should differential display not answer the questions we are posing; however, for the sake of brevity and since they have not yet been pursued, I will not mention them further in this report.

3. Examination of a possible association between abnormal glucose transport and the neoplastic phenotype.

Glucose transport in MCF-7 breast cancer cells, which exhibit polarized expression of membrane glycoproteins(Zou, et al., 1989), will be characterized by the methods described above. This will include comparisons of hormonal responsiveness of glucose transport. This observational study will describe differences between two established cell lines, the CIT_3 and MCF-7 lines, but differential expression of transporters or other regulators between the two lines, while suggestive, cannot prove the importance of a given protein.

Brown et al.(Brown and Wahl, 1993) examined twelve breast tumors and showed by immunohistochemistry that higher expression of the glucose transporter GLUT1 by breast cancer cells compared with the healthy breast tissue is common. However, these investigators did not quantitatively assess glucose transporter targeting. I intend to carry out these studies quantitatively, at the level of mRNA as well as protein, in order to understand the magnitude of the changes in glucose transport seen in neoplasia. Tom Wheeler, M.D., of the Department of Pathology at Baylor College of Medicine, is also collaborating with me in this aspect of the study; the up to 20 breast cancer specimens per year referred to above will also be studied to determine patterns of glucose transporter expression in breast cancer. I anticipate studying at least 20 specimens before drawing conclusions.

The observational approach outlined in the previous paragraph can not distinguish whether changes in glucose transporter expression, which are, after all, likely to be observed, are central and necessary phenomena, or simply epiphenomena. The relationship between expression of a specific glucose transporter and the neoplastic phenotype will therefore be directly tested by stable transfection as described above. Normal mammary epithelial cells will be stably transfected with expression vectors containing the non-inducible CMV promoter, and will express the heterologous

transporter constitutively. This will directly test the link between the transporter in question and changes in phenotype, including altered transport properties, glucose utilization, synthesis of milk components, growth characteristics, and cellular morphology. Overexpression in neoplastic cells of factors which might alter glucose transporter activity will directly test the importance of elevated glucose transport activity in contributing to tumor growth. Glucose transporter levels can also be reduced up to 80% using antisense RNA methods(Valera, et al., 1994), providing another avenue for determining the significance of GLUT1 overexpression for the neoplastic mammary cell phenotype. An expression vector based on the mouse mammary tumor virus promoter, which is active in mammary gland(Gunsburg and Salmons, 1986), confers highly inducible synthesis of heterologous proteins in epithelial cells(Hirt, et al., 1992). This will be useful in ruling out any effects of constitutive expression on membrane trafficking or differentiation, and in establishing more firmly the link between expression of heterologous protein and changes in phenotype.

An elegant method for reconstitution of mouse mammary gland from mammary epithelial cells(DeOme, et al., 1959, Medina, 1973) has been adapted to cells genetically altered in vitro(Edwards, et al., 1992) in order to study physiological and morphological correlates of oncogene expression. Normal mammary epithelial cells are isolated from one mouse and briefly put into primary culture, where a gene is introduced by retroviral infection; the cells are then transplanted into the mammary fat pad of a mouse from which the endogenous epithelium has been removed. The transplanted cells grow to reconstitute a "tree" of glandular epithelium. Transplants carrying the Wnt-1 oncogene grew in a hyperplastic pattern, showing abundant fine side-branches, but without development of alveoli. The same authors also showed that expression of the neu/erbB-2 oncogene induced epithelial abnormalities similar to human atypical hyperplasia and sclerosing adenosis(Bradbury, et al., 1993). This will be a useful method to study specifically in vivo the consequences on cellular organization and function of the overexpression of transporters or other unique factors identified in the course of this study. I will also explore the possible use of this system to understand the impact of these genetic alterations on lactose synthesis in vivo. The results of experiments with reconstituted mammary glands will serve to validate, or to question the significance of, findings from established cell lines or tumors.

Body

Experimental methods and procedures

Subcellular fractionation- (Haney, et al., 1991)- Glands were removed and homogenized in PBS (5 cc/g) with 1 mM EDTA as described above. Centrifugation for 10 min at 3000 g produced a pellet, which was resuspended and centrifuged again at 3000 g. This 3000 g pellet is the nuclear pellet. The combined supernatants of the 3000 g centrifugations were centrifuged for 10 min at 17000 g. This 17000 g pellet is the light mitochondrial pellet and is enriched in Golgi. This pellet was subjected to a self-generating iodixanol density gradient (10%-37%) by combining with a 15% iodixanol solution and centrifuging for 3 h at 180000 g. Fractions were collected using a Labconco Auto-Densiflow collector. Aliquots containing 20 µg of protein were subjected to Western blotting as described above.

Western blots- (Haney, et al., 1991; Haney and Mueckler, 1994)- Homogenates were prepared as described above and solubilized in 1%SDS. Samples were subjected to SDS-polyacrylamide gel electrophoresis, with 20 µg protein per lane. Samples were not be boiled before loading, since this distorts electrophoresis of membrane proteins. Purified human erythrocyte GLUT1 supplied by Dr. Mueckler served as standard. Proteins were transferred to nitrocellulose. Membranes were treated with Blotto for 30 min and exposed to primary antibody for 1 h. Primary antibody was a 1:1000 dilution of the highly specific, well-characterized F350, directed against the final 16 amino acids of the C-terminal cytoplasmic tail of GLUT1. Blots were washed three times for ten minutes each with PBS containing 1%SDS. Secondary antibody was horseradish peroxidase-antirabbit IgG, and signal was developed by the Amersham ECL protocol. Relative protein levels were determined by densitometry. Results shown are representative of two to three independent studies per timepoint.

Immunofluorescent microscopy- (Haney, et al., 1991; Haney and Mueckler, 1994)- Frozen tissue sections were prepared. This is the gentlest method available and preserves cell structure and antigens. A 0.5 cm x 0.5 cm x0.5 cm piece of gland was dissected and frozen gradually in Lipshaw Number 1 solution, and stored at -70°C. Using a cryostat, 5-10 µm sections were prepared. Sections were air-dried, dipped in paraformaldehyde for 2 min, washed in PBS, and placed in 1%NP40, PBS, for 5 min, then rinsed several times in PBS. Sections were exposed to peptide-affinity purified GLUT1 antibody at a concentration of up to 5 µg/ml overnight at 4°C in a humidified chamber. Through careful selection of antibody concentrations to be used, the possibility of non-specific staining was minimized, then ruled out using appropriate controls, include antibody preabsorbed with the antigenic peptide. Sections were washed three times for 5 min each in PBS with 1% Triton X-100. Secondary antibody was

FITC-labeled anti-rabbit IgG $F(ab)_2$ in PBS, 0.1% horse serum, applied at the recommended concentrations (Organon Teknika) for 1 h at room temperature. Sections were rinsed three times for 5 min each in PBS with 1% Triton X-100. One drop of Vectashield anti-photobleaching agent was applied, then coverslips were placed. Specimens were viewed using a Olympus ix70 microscope equipped for fluorescence. Localization of signal to basolateral membrane, apical membrane, Golgi membrane, and/or other intracellular compartments was assigned by surveys of low- and high-power fields.

Differential display- (Zhang and Medina, 1993) RNA was extracted using standard procedures, including tissue homogenization, chloroform treatment, and isopropanol precipitation. Poly A+ mRNA was isolated independently from CIT3 cells grown in growth medium and secretion medium, using the FastTrack mRNA isolation kit, and cDNA was synthesized. Reverse-transcribed cDNAs were amplified by PCR using arbitrarily chosen primers (Display Systems). 5 µg of lactating mammary gland poly(A)+ RNA was incubated with 10 U of reverse transcriptase for 1 h to synthesize first-strand cDNA. After incubation with 0.1 M NaOH for 20 min at 65°C, single strand cDNA was phenol/chloroform extracted and ethanol precipitated. Approximately one-fourth of the cDNA produced was used per PCR reaction, with oligonucleotides at 4 µM each. PCR were carried out for 25 cycles of denaturation at 100°C for 2 min, annealing at 28°C for 30 sec, and extension for 10 min at 28°C . Amplified DNA was phenol/chloroform extracted, ethanol precipitated, and subjected to agarose gel electrophoresis. Fragments were subcloned and sequenced.PCR products were analyzed on agarose gels, and reproducibly upregulated bands were selected for further study. These cDNAs were subcloned into TA vectors and sequenced. These inserts and PCR primers selected from them were used to screen a lambda-gt11 library from mouse mammary gland at day 10 of lactation.

Northern blots- RNA from cell culture or mammary gland was denatured with formaldehyde, separated on an agarose gel, and transferred to nylon filters. Hybridizations with 32P-labelled oligonucleotides or cDNAs were carried out at between 42 and 55 degrees centigrade. Filters were washed and subjected to autoradiography.

Library screening- This overview describes screening the commercially available lgt11 expression library from lactating mouse mammary gland (Clontech), which has more than 10^6 independent clones, 32P-labelled cDNA or oligonucleotide. Space restrictions prohibit step-by-step details. This procedure would be modified for screening using radiolabeled peptides. Approximately 10^4 plaques per 90 mm plate were plated. After lytic phage growth, a dry nitrocellulose filter was placed on each plate and incubated at 37°C for 3.5 h. Filters were rinsed and probed. Filters were washed and subjected to

autoradiography. The positive plaques were removed from the original plates and the screening procedure was repeated until all plaques are positive. DNA was isolated from lambda lysates, and inserts were excised, cloned, and sequenced. Clones were sequenced.

Assumptions

Major assumptions include specificity of the antibody, the reliability of marker enzymes in the indication of organelle distribution, and the attribution of signal on immunofluorescent cell staining to proteins of different molecular weight which are recognized by the GLUT1 antibody. The first of these has been assured by careful analysis of this antibody over several years and by the use of peptide-affinity purified antibody to reproduce the results, and by the use of appropriate controls. The second has been somewhat confusing, and we are still studying the distribution of different marker enzymes in the gradient. We do use the refractive index of individual gradient fractions to characterize them, and identify as Golgi those fractions with the characteristically low density (1.06-1.09 g/cm3) physical property of Golgi. The final concern is more a caveat than an assumption, since we know from our Western blots that the antibody specifically recognizes proteins of MW 50-80 kD, all of which would generate an equivalent signal on a per molecule basis when viewed by immunofluorescence

Screening an expression library with GLUT1 antibody in an effort to clone p72 would result in cloning GLUT1 as well, increasing the effort required to specifically identify a p72 clone. More generally, some putative would undoubtedly represent GLUT1, but some should also represent a novel glucose transporter, provided homology does indeed occur.

Successful characterization of the subcellular localization of novel proteins will require the production of specific antibody. Alternatively, epitopes may be engineered into novel proteins by cloning them into the cDNA to allow the use of sensitive and specific antibodies. This strategy was employed in my previous work (Haney, et al., 1995), in which a species-specific epitope was engineered into chimeric glucose transporters to enable their specific detection.

Results and discussion

In work funded by this grant and reported in previous annual reports, double-label immunofluorescence and subcellular fractionation by density gradient centrifugation were used to demonstrate that GLUT1 is localized, both in vitro and in vivo, in the Golgi in response to the hormonal milieu of lactation. Northern and Western blots for GLUT1 and GLUT5 indicated that the developmental regulation of glucose transporters was isoform-specific, and a

rapid decline in GLUT1 levels at weaning was linked to changes in the translational efficiency or increased GLUT1 degradation.

Therefore, during the reporting period, I focused on the weaning period, since it offers the opportunity to examine whether transporter targeting is altered at a point where it is imperative that milk production be rapidly curtailed. Failure to observe changes in glucose transporter targeting would suggest that this is not an important component of the mechanism which regulates milk production. We have also begun to assess the virgin and late pregnant mouse mammary gland.

To examine the regulation of GLUT1 targeting during lactation, mouse pups were prematurely weaned at 18 days of age, the peak of milk production. This tests the hypotheses that GLUT1 is targeted to Golgi during lactation, and that its regulation during weaning would be consistent with an important role in the regulation of lactose synthesis. Subcellular fractionation and density gradient centrifugation were employed, as described in IV.A.4.c, to isolate a Golgi-enriched fraction of mammary gland. Enrichment in Golgi but not plasma membrane was verified using galactosyl transferase as a Golgi marker and 5'-nucleotidase as a plasma membrane marker (data not shown). GLUT1 was quantitated by Western blot analysis. Experiments were done in duplicate or triplicate, and representative results are shown. As shown in **Figure 1**, *GLUT1, at 43 kD, is 5-fold enriched in the Golgi fraction of lactating mammary gland* compared to total cellular homogenate. Enrichment continued after 3 h of weaning, but *was lost by 5 h of weaning*, and diminished further thereafter. Enrichment could be restored by returning the pups to the mother for 5 h , and moreso after 15 h. A second effect of weaning, seen after 10 h, is that total cellular content of GLUT1 begins to decrease. **Figure 2** shows the *decline in total cellular and Golgi GLUT1 that occurs during natural weaning between 18 and 29 postnatal days*. Taken together, the data confirm the validity of our previous observations in mammary epithelial cells in culture and suggest that GLUT1 may be an important regulator of lactose synthesis and milk secretion.

Unexpectedly, the GLUT1 antibody also identified higher MW proteins. *A 72 kD protein showed even more striking Golgi enrichment than GLUT1, and also showed loss of Golgi enrichment during weaning* (**Figures 1 and 2**). Intermediate MW forms at 50 and 65 kD were also observed. The 50 kD and 65 kD forms were enzymatically deglycosylated using endoglycosidase F to generate aglyco-GLUT1 (data not shown). As shown in an individual density gradient result from a 5 h wean, then 15 h return of pups (**Figure 3**), *the 50 kD and 65 kD forms , which represent alternatively glycosylated GLUT1, each display specific patterns of appearance, disappearance, and subcellular localization*. For example, the 65 kD form is found only in fractions 9-12. This shows that the degree of GLUT1 glycosylation is an important determinant of its subcellular targeting.

Interestingly, as also shown in **Figure 4**, *the 72 kD protein was resistant to enzymatic deglycosylation*. Note that while GLUT1 itself is deglycosylated to a 38 kD form, the 72 kD protein was not affected by this treatment. Because the 72 kD protein also was degraded within 5 h of weaning, coincident with the appearance of an 80 kD protein, an anti-ubiquitin antibody was also used for immunoblotting. Ubiquitin is an 8 kD protein important in regulation of protein degradation. **Figure 5** represents the density gradient observed after 3 h of weaning; the upper panel shows immunoblotting with antibody to GLUT1, while the lower panel was immunoblotted with antibody to ubiquitin. The data show that the *80 kD protein is the major ubiquitin-containing protein present in these fractions*, and presumably represents the ubiquitin-conjugated 72 kD protein. An important control, repeating all these experiments using antibody preabsorbed with the antigenic peptide used to generate the GLUT1 antibody, gave no signal (data not shown). This proves the specificity of the antibody for all forms identified.

In the remainder of this report, I refer to the 72 kD protein as p72. p72 is unique to Golgi of lactating mammary gland, is highly enriched in Golgi, and is rapidly and specifically degraded upon weaning. However, although it shares the GLUT1 C-terminal antigenic determinant, it is not a glycoprotein and it presumably consists of about 700 amino acids. The known facilitated diffusion glucose transporters are glycoproteins consisting of 492-527 amino acids. Therefore, p72 is a good candidate for a regulator of glucose transporter targeting, but not for a glucose transporter itself.

Studies of virgin and late-pregnant animals were carried out after the end of the reporting period and will be discussed in next year's report.

We have also performed immunostaining for GLUT1 in virgin, late-pregnant, lactating, and weaning mice to demonstrate specific targeting of GLUT1 by an independent method. The images have not been captured due to technical issues related to computer compatibility as a consequence of my moving from St. Louis; this problem will be overcome shortly. I can report that inspection showed strong basolateral staining of GLUT1 in lactating mice, but much weaker staining in virgin mice. The intracellular staining was less than expected, and we are preparing to check other markers to see if this simply reflects inadequate permeabilization of the cell to provide antibody access to antigen, or whether there is indeed a discrepancy between the methods of immunohistochemistry and density gradient centrifugation. Interestingly, there is also very little staining for GLUT1 in late pregnant mice, suggesting that the expression of high levels of GLUT1 must be a perinatal phenomenon, and strengthening the case for glucose transport as an important regulator of lactose synthesis and milk production. We already have preliminary evidence and we will be working to confirm this impression with further immunocytochemistry as well as by the

independent method of Western blotting, and we plan to delineate the time course of GLUT1 induction during the perinatal period in an analagous manner to what we have done during weaning.

We have concentrated on differential display as the method of choice for pursuit of Task II, the identification of novel proteins involved in glucose transport in lactating mammary epithelia. This has involved the use of nine downstream anchored primers and 24 arbitrarily chosen upstream primers for 216 PCR reactions, studying genes differentially expressed in CIT3 cells in the presence of the lactogenic hormones prolactin and dexamethasone. Our initial analysis, of products of four of the downstream primers, revealed 12 upregulated and 22 downregulated products. Beginning with independently prepared mRNA samples, 7 of the upregulated products and 9 of the downregulated products were found to be reproducible. A repeat analysis resulted in confirmation of 8 of the upregulated products and 12 of the downregulated products. Since genes involved in altering glucose transporter targeting should be upregulated in this setting, we focused on these 8 upregulated products. All 8 were reamplified. Of these, five showed upregulation by Southerns , using 32P-labelled cDNA from cells grown in the presence (secretion medium, SM), or the absence (growth medium, GM) of prolactin and dexamethasone. Initial results were confusing due to the inaccuracy of RNA quantitation, presumably due to milk contamination. To overcome this, we used GAPDH as a control for the amount of cDNA added to the gel (**Figure 6**).

Highest priority was assigned to a clone we called "clone H", which is capable of coding for a sequence remarkably similar to the trans-Golgi targeting region of the GLUT4 glucose transporter. Of the five clones, this was the only one with an apparent homology to glucose transporters. Thus, although as shown in **Figure 6**, its induction in secretion medium was not nearly as great as initially thought, it is still of great interest. This is reinforced by its expression in mammary gland of lactating mice (**Figure 7**).

This "clone H" cDNA was subcloned into a TA vector. The clone H cDNA was used to screen a lambda-gt11 phage library using conventional plaque-lift methods. Initial library screens were negative. Library screening was repeated using higher specific activity probes and a positive clones was identified. This was used in attempts to isolate full-length clones from the lambda-gt11 library. Screening was done using PCR of lambda-gt11 pools using a clone H-SP6 oligo probe. Positive pools were reprobed using clone H cDNA. HI and HII, small and large inserts respectively, were identified. A single plaque containing HII, the large insert, was isolated. PCR resulted in only one fragment, which was sequenced. The 1 kB sequence is shown as **Figure 8** . A Genbank search of this sequence (**Figure 9**) reveals interesting homologies of uncertain significance, including to cAMP respone element binding proteins, tumor necrosis factor, and

glucokinase, and confirms that the clone represents a previously unknown gene. Efforts to assemble a full length clone continue.

Clone 5(8) hybridized with GM, SM, and RNA from lactating day 10 mice. Clone D6(9) hybridized with GM, SM, and RNA form lactating day 10 and 17 and weaning day 10. Clone D6(14)B and clone #63 hybridized with GM, SM, and lactating and weaning mice. Clone H hybridized with GM and SM. A screening process as described for clone H has been applied to these other novel sequences. For clone #63, this has identified two pools of lambda-gt11 which contain positive clones, and screening continues. The analogous process with clone D6(14)B has also identified two pools of lambda-gt11 which contain positive clones, and screening continues. The analogous process with clone D6(9) has identified one pool of lambda-gt11 which contains positive clones, and screening continues. Screening has not yet been started for clone 5(8).

Conclusions

1. There is enrichment of Golgi by GLUT1 during lactation. This continues after 3h of weaning, but is lost by 5h of weaning, and diminishes further thereafter.

2. Enrichment can be restored by returning the pups to the mother for 5h.

3. A second effect of weaning, seen after 10h, is that total cellular content of GLUT1 begins to decrease.

4. Unexpectedly, higher MW proteins were also identified by the GLUT1 antibody. A 72 kD protein showed even more striking Golgi enrichment than GLUT1, and also showed reversible loss of Golgi enrichment during weaning.

5. Intermediate MW forms at 50 and 65 kD were also observed. These each demonstrate specific patterns of appearance, disappearance, and subcellular localization, and can be deglycosylated to give aglyco GLUT1. The degree of GLUT1 glycosylation is an important determinant of its subcellular targeting.

6. The 72 kD protein was resistant to deglycosylation. Based on the kinetics of its appearance and disappearance, its physicochemical properties which suggest it is not a glucose transporter but may be capable of associating with GLUT1 based on sharing of the C-terminal epitope, and based on its subcellular localization in the Golgi, p72 represents an excellent candidate for a protein involved in sequestering glucose transporters within the Golgi.

7. Ubiquitin appears to play an important role in the rapid degradation of GLUT1 and p72 during premature weaning.

8. In mice, when nursing is interrupted for as little as five hours, the targeting of GLUT1 to Golgi is rapidly but reversibly lost. After as little as ten hours, total cellular GLUT1 content begins to decrease. These changes should result in the reduced availability of glucose within the Golgi, and therefore a reduction in the rate of lactose synthesis and milk production. We can not yet comment on the kinetics of this process in human mammary epithelial cells. However, we speculate that the targeting of GLUT1 to Golgi is an important requirement for successful human lactation. I continue to believe that the identification of the cellular proteins, perhaps including p72 and ubiquitin, that constitute the mechanism by which these changes in glucose transporter targeting and amount, respectively, are achieved is a necessary step if we are to take advantage of this mechanism to alter glucose transporter targeting in cancer cells.

9. Differential display analysis of gene expression under regulation by of prolactin and dexamethasone has revealed several novel genes, which we are in the process of identifying. Most progress has been made on "clone H", which may code for a motif

remarkably similar to the trans-Golgi targeting motif of GLUT4. This is the best candidate of this group, based on the limited information available thus far, for a gene involved in regulation of glucose transport in lactating mammary gland. Efforts continue to isolate full length clones for all novel genes identified thus far.

Recommendations in Relation to Statement of Work

I am satisfied with the Statement of Work as currently written, with the exception that I feel for reasons outlined above that working in mice has several advantages over working in rats. We have examined the weaning period and do intend to examine the perinatal period in greater detail than outlined in the Statement of Work. As noted in the Prologue, work is just now underway on the third task. I do feel that accomplishing all tasks on the Statement of Work is feasible.

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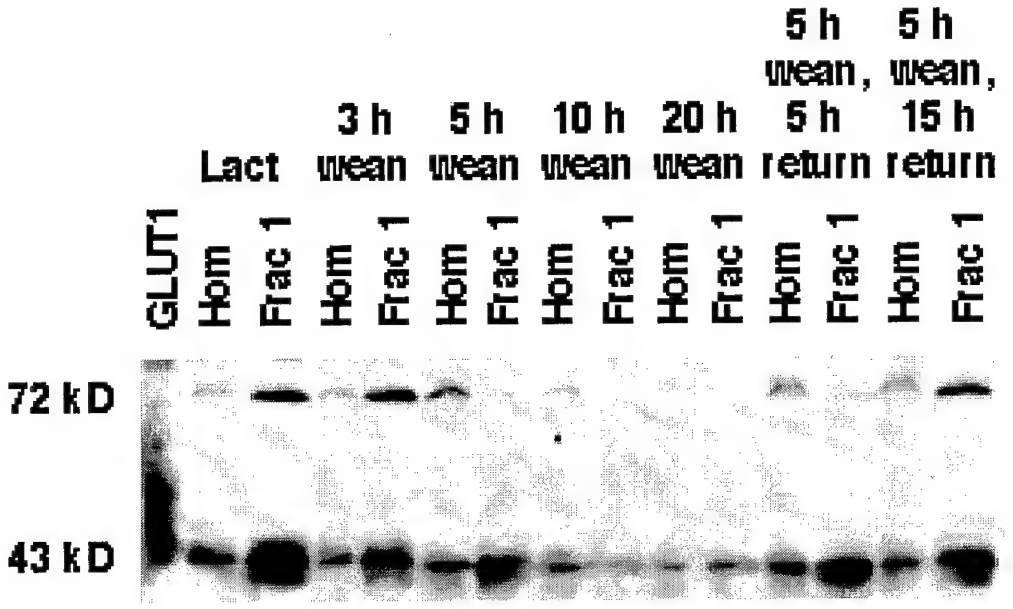


Figure 1, *GLUT1, at 43 kD, is 5-fold enriched in the Golgi fraction of lactating mammary gland* compared to total cellular homogenate. Enrichment continued after 3 h of weaning, but *was lost by 5 h of weaning*, and diminished further thereafter. Enrichment could be restored by returning the pups to the mother for 5 h. A second effect of weaning, seen after 10 h, is that total cellular content of GLUT1 begins to decrease.

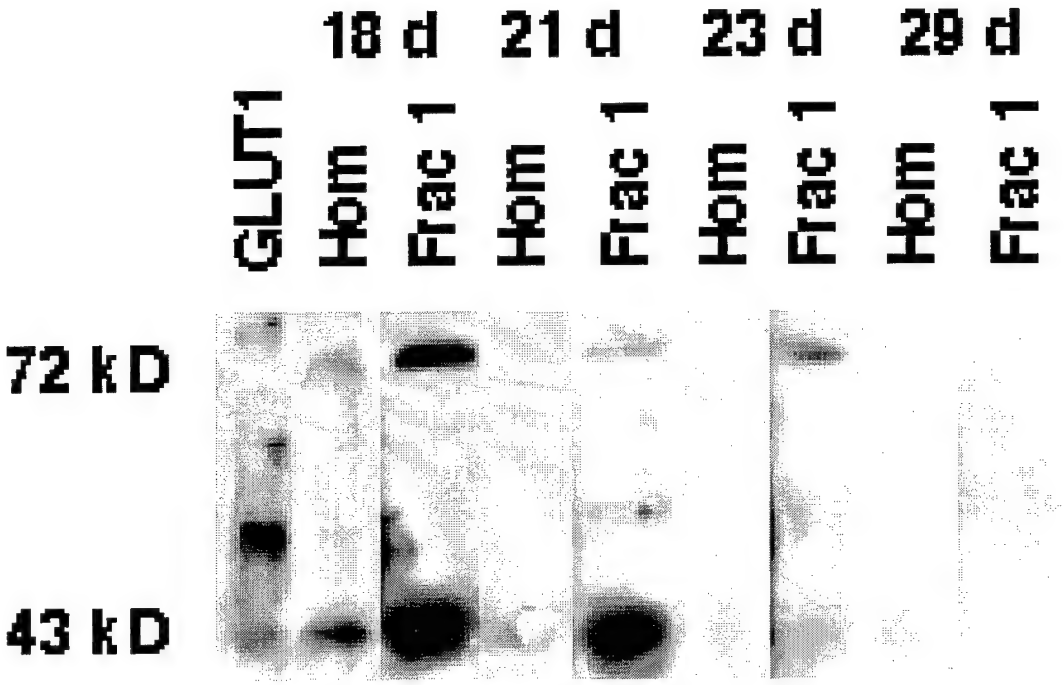


Figure 2 shows the *decline in total cellular and Golgi GLUT1 that occurs during natural weaning between 18 and 29 postnatal days.*

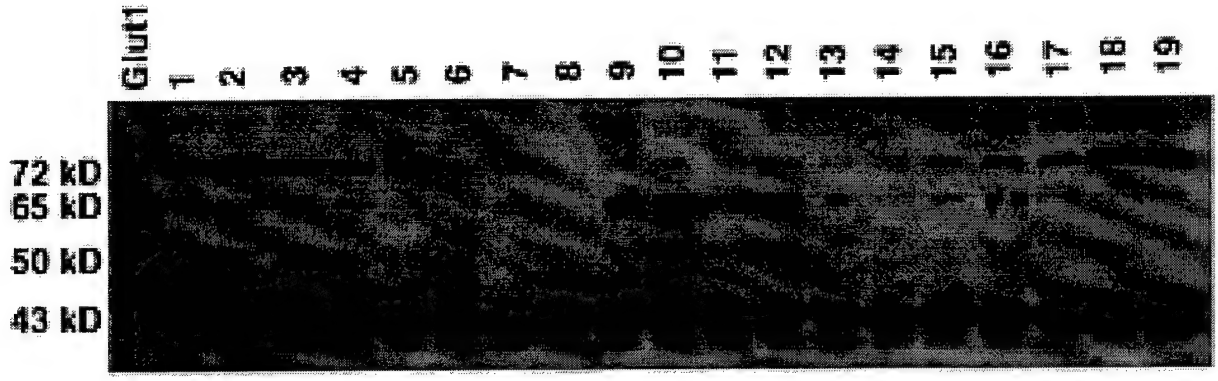


Figure 3 As shown in an individual density gradient result from a 5 h wean, then 15 h return of pups, *the 50 kD and 65 kD forms , which represent alternatively glycosylated GLUT1, each display specific patterns of appearance, disappearance, and subcellular localization*. For example, the 65 kD form is found only in fractions 9-12. This shows that the degree of GLUT1 glycosylation is an important determinant of its subcellular targeting.

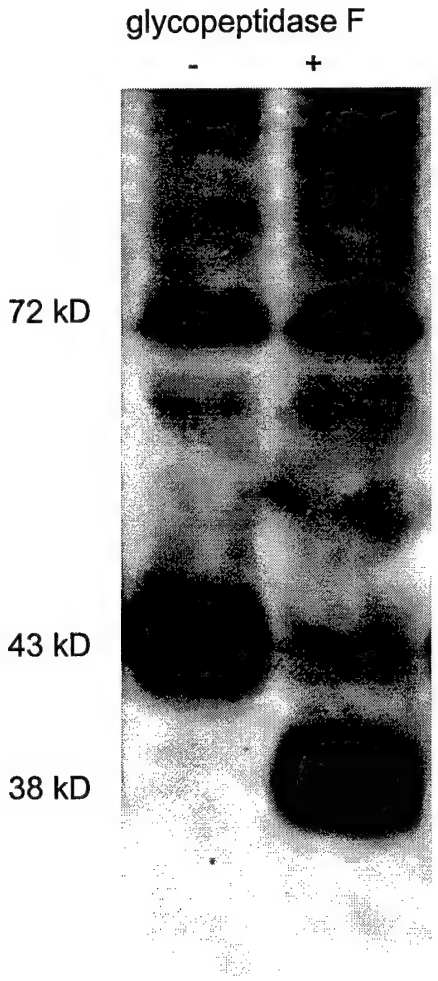
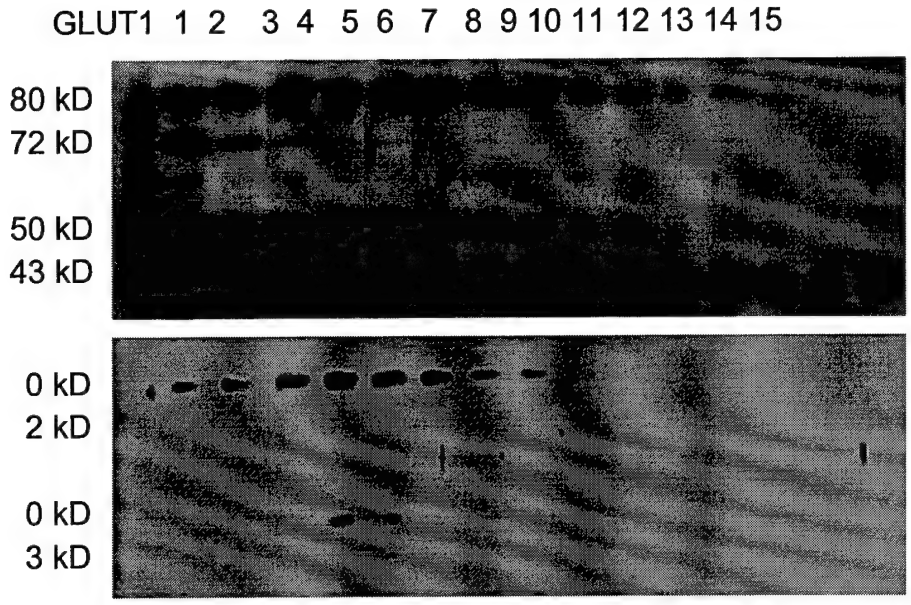


Figure 4. *The 72 kD protein was resistant to enzymatic deglycosylation.* Note that while GLUT1 itself is deglycosylated to a 38 kD form, the 72 kD protein was not affected by this treatment.



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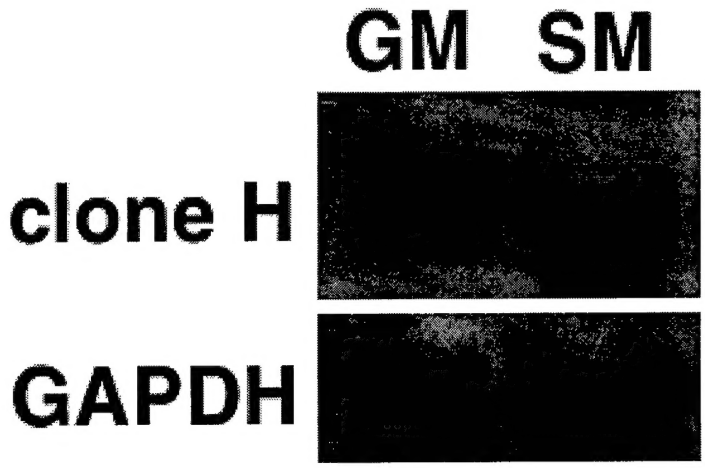

Figure 6. Apparent induction of clone H in SM is largely due to inequalities in loading of RNA on the gel, as shown by GAPDH as control.

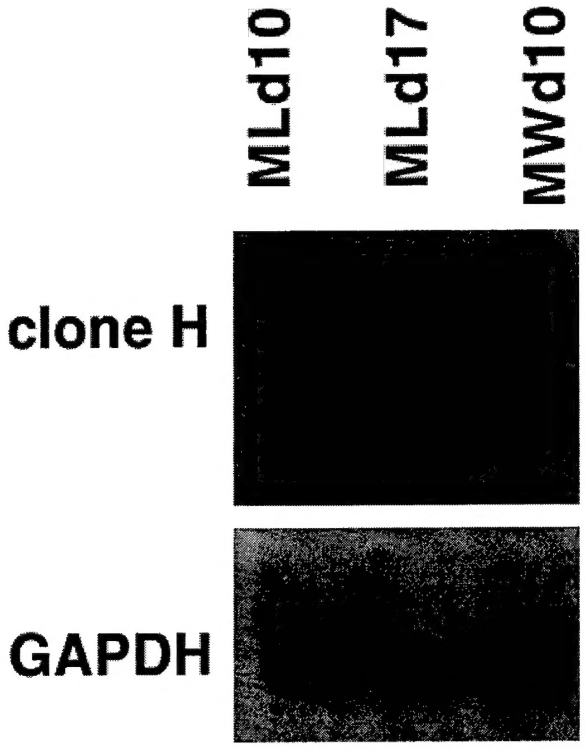

Figure 7. Clone H detects an mRNA expressed in lactating mouse mothers on days 10 and 17, and in mothers already weaned for one day on day 10 of lactation. Northern blot, GAPDH as control.

GGAATTCCGCACCANNTTACCTGCATGGGTATTTCCATTCAATACAATGA
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CAATGGCACAAGCAATGCCACAGCCATCTCGTATAAACCACATGNTCCCGCAG
GACCAGGGAAGGGAGGTGGGGCACACTTCTCTGGCTGGAGTACTCTGGTTTCC
GCTCAATGTCTCGGAAGTGATGGGTGGGGATAAGCATCATAAGCTATTCTGTCC
ATACTGCATCTGAAAGGAAAGGA

Figure 8. 1044 bp sequence of clone H.

```
BLASTN 1.4.9MP [26-March-1996] [Build 14:27:07 Apr  1 1996]


Query=  clone H
        (1044 letters)

Database:  Non-redundant GenBank+EMBL+DDBJ+PDB sequences
           299,613 sequences; 545,122,873 total letters.
```

Sequences producing High-scoring Segment Pairs:		High Score	Smallest Sum Probability P(N)	N
emb|X15875|HSCREBP1	Human mRNA for cAMP response eleme...	160	0.017	1
emb|X07808|MMB1R	Mouse mRNA with B1 repetitive elem...	121	0.039	2
emb|X62249|MMSCRNA	M.musculus scRNA	112	0.33	2
emb|Z81450|HS333B10	Human DNA sequence from cosmid 333...	143	0.37	1
emb|Y00137|MMTNFBG	Mouse tumor necrosis factor-beta (...	115	0.71	2
gb|AC002478|AC002478	Human BAC clone GS002F01, complete...	136	0.72	2
gb|M17015|MUSLTA	Mouse LT lymphotoxin (LT) gene, co...	115	0.72	2
emb|Z66065|HS73G2F	H.sapiens CpG DNA, clone 73g2, for...	136	0.73	1
emb|Z82171|HSB11B7	Human DNA sequence from cosmid B11...	124	0.83	2
gb|L41631|MUSGLUCA	Mus musculus glucokinase gene, com...	137	0.88	2

```
emb|X15875|HSCREBP1 Human mRNA for cAMP response element (CRE-BP1)
            binding protein
            Length = 1647

  Plus Strand HSPs:

 Score = 160 (44.2 bits), Expect = 0.017, P = 0.017
 Identities = 44/59 (74%), Positives = 44/59 (74%), Strand = Plus / Plus

Query:   290 CCTGTGTAAGCTAAATGAAGACTAAACCTATCCATGTCCTGAAACTGGATGTGGCCTGC 348
             ||||||  ||  | | ||| ||| || ||| || |||| |||   ||||||||||| ||
Sbjct:    71 CCTGTGGAATATGAGTGATGACAAACCCTTTCTATGTACTGCGCCTGGATGTGGCCAGC 129
```

Figure 9. Genbank search of clone H.